

Potential of dry matter yield from alfalfa germplasm in composing base populations

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Abstract: The aim of this study was to obtain estimates of genetic parameters via meta-analysis and mixed models to evaluate the potential of the germplasm from Embrapa Pecuária Sudeste for integration in breeding programs. Data on the dry matter yield of 77 accessions evaluated in 24 harvests were used. Descriptive analysis was performed for mixed model and meta-analytical estimates. The variances for each parameter were estimated. For the meta-analysis, variances were used as weighting measures, and the fixed and random models were tested. A diagonal structure was considered for the residual variance matrix. Mean dry matter yield ranged from 1019.5 to 2684 kg ha⁻¹. The phenotypic, permanent environmental, and genotypic variances oscillated widely throughout the harvests, as well as heritability. Meta-analytical estimates proved to be an accurate and complementary approach to the mixed model analysis. Both mixed model and meta-analytical estimates indicated the breeding potential of the alfalfa germplasm.

Keywords: *Medicago sativa* L., mixed models, meta-analysis, genetic estimates, longitudinal data.


INTRODUCTION

The leguminous plant alfalfa (*Medicago sativa* L.) is one of the most important staple forage crops in temperate countries (Ferreira et al. 2008, Annicchiarico et al. 2015). It is increasingly adopted in Brazil because of characteristics such as high protein content (Ferreira and Vilela 2015). Embrapa Pecuária Sudeste has an alfalfa germplasm collection composed of 77 accessions of temperate genetic background. The company performs routine evaluations of its germplasm in order to compose synthetic populations well adapted to Brazilian edaphic and climatic conditions. Plant breeding is a very expensive process that requires time, work, and investment. A wrong decision may affect the breeding program and may be irreversible in the short term (Streck et al. 2018). Therefore, it is essential to estimate genetic parameters as accurately as possible to understand the effects of genes, select promising accessions, and predict gains from selection.

Estimation of variance components allows prediction of genotypic variance from phenotypic information (Cruz et al. 2012). This information reflects the genetic merit of the germplasm and is fundamental in any breeding program, since genetic gains can only be obtained if there is genetic variability. Heritability reflects how much of the phenotypic variation observed in a trait is due to

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genetic effects. Heritability guides breeders in how a breeding program can be conducted (Ramalho et al. 2012). The mean is another parameter that defines the merit of a population in a breeding program. There is no advantage in having genetic variability for a given trait if the mean of phenotypic values is below expectations.

A set of experiments carried out over time generates an individual information set that requires special attention. Data sets of this nature are commonly analyzed through combined analysis or through a meta-analysis approach (Lin and Zeng 2010). It should be emphasized that data from alfalfa experiments are classified as longitudinal data, also referred to as repeated measures data. Longitudinal data are characterized when two or more observations or measurements are collected on the same individual over time (Fitzmauric et al. 2008). Longitudinal data can be evaluated through models, which consider error dependence to obtain genetic estimates of each harvest time, or through meta-analysis, which combines the individual estimates of the parameters in a single measure. Both approaches provide different perspectives on the parameters. Through a mixed model approach, it is possible to improve the accuracy of genetic values, investigate how well genotypes perform under different environmental conditions, and evaluate the trajectory through time, considering proper modelling of residuals (Campbell et al. 2019).

Meta-analysis is used to summarize individual results in a single measure (Engels et al. 2000), known as a combined estimate or a meta-analytical estimate. An upside of this approach is assignment of different weights to each study or experiment, based on their accuracy. The weight is generally equivalent to the inverse of the variance of a given parameter (Fagard et al. 1996). Meta-analytical estimates can consider the effect of any particular aspect that occurs in an experiment (e.g., environments, harvests, years), due to the possibility of examining the variability among the combination of these factors. Meta-analysis technique proved most appropriate to evaluate long-term parameters in breeding programs, for not only taking into account the average of the parameters, but also their variation in the groups over the course of time (Costa et al. 2020).

A satisfactory mean and high genetic variability of the base population is necessary for the success of any breeding program. This will ensure continuous genetic progress throughout the various selection cycles (Maluf and Ferreira 1983, Fialho et al. 2020). In this study, we investigated whether individual estimates of genetic parameters could be comparable to meta-analytical estimates to guide breeders in choosing germplasm to begin an alfalfa breeding program. The aim of this investigation was to obtain meta-analytical estimates using genetic parameters and to evaluate the potential of germplasm from Embrapa Pecuaria Sudeste to compose base populations.

MATERIAL AND METHODS

Data Collection

Data from 24 alfalfa harvests were used, which included data from 77 genotypes from the Instituto Nacional de Tecnología Agropecuaria (INTA) – Argentina. The experiments were conducted in the experimental field of Embrapa Pecuaria Sudeste, São Carlos, São Paulo (lat 21° 57' 42" S, long 47° 50' 28" W, alt 854 m asl), in a randomized complete block design, with three replicates. Each experimental unit consisted of four 4.0-m rows, with row spacing of 0.2 m. The two central rows, eliminating 0.5 m from each end, were used for data collection.

Soil tillage consisted of plowing and two passes with a disk harrow. Lime and fertilizers were broadcast on the planting area, according to soil chemical analysis. Triple superphosphate was applied as a P source, potassium chloride as a K source, and FTE BR-12 as a micronutrient source. Seeds were inoculated with strains of *Rhizobium meliloti* - SEMIA 116. After each harvest, the plants received cover fertilization according to soil analysis. The alfalfa was irrigated, except for harvests 8, 9, 10, 19, 20, and 21, for which irrigation was suspended. Irrigation was performed by a central pivot, with management based on the difference between evaporation and rainfall, according to Rassini (2002). Weeds were controlled by a single application of Pivot, at a dosage of 1 L ha⁻¹, and Fusilade, at a dosage of 1.5 L ha⁻¹ (Brighenti and Castro 2008). Dry matter yield (kg ha⁻¹) was obtained by manually cutting the plants at eight to 10 cm above the ground when each cultivar reached the flowering stage.

Estimation of genetic parameters

The mean (μ), phenotypic variance (Pheno.V), genotypic variance (Geno.V), permanent environmental variance

(Perm.Env.V), and heritability (h^2) were estimated for each cutting based on the following mixed model:

$$y = Xb + Zg + Wp + e$$

where y is the vector of phenotypic data, b is the vector of the fixed effect of replication, g is the random vector of genetic effects, p is the random vector of the permanent environment, and e is the random vector of residuals. X , Z , and W are incidence matrices for fixed, genetic, and permanent environmental effects, respectively. A diagonal structure for the matrix of residual variances was considered, so each harvest had a specific residual variance. The BLUPs for each genotype within each harvest were obtained, as well as estimates for Pheno.V, Geno.V, Perm.Env.V, and h^2 . The variances for each random effect were obtained by restricted maximum likelihood (REML). ASReml (Gilmour et al. 2015) was used to fit the mixed model. The variances associated with the estimates were obtained as follows:

$$V_x = \sum_{i=1}^n \frac{(x_i - \bar{x})^2}{n-1}$$

where V_x represents the variance of estimate X , x_i is the mean of the accessions in the i th harvest, \bar{x} is the overall mean, and n is the number of harvests.

Meta-Analysis

The weight adopted for each parameter was given by the respective variance estimates. The heritability variances were estimated according to Falconer and Mackay (1996). The variances associated with the estimates of the mean (μ), phenotypic variance (PV), and genotypic variance (GV) were obtained according to Cruz et al. (2012):

$$V(\mu) = \frac{MS_e}{rg} \quad V(\hat{\sigma}_p^2) = \frac{1}{r^2} \left[\frac{2(MS_g)^2}{(g-1)+2} \right] \quad V(\hat{\sigma}_g^2) = \frac{2}{r^2} \left[\frac{MS_g^2}{g+1} + \frac{MS_e^2}{(r-1)(g-1)+2} \right]$$

where r represents replicates, g is the number of treatments (alfalfa accessions), MS_e is the mean square of residuals, $\hat{\sigma}_p^2$ is the estimate of PV, MS_g is the mean square of treatment with $(g-1)$ degrees of freedom, and $\hat{\sigma}_g^2$ is the estimate of genotypic variance.

Meta-analytical estimates for the parameters summarized the results of k harvest obtained by two regression models (fixed and random) using maximum likelihood estimation (MLE). The fixed regression model presupposes homogeneity between parameter estimates, while the random regression model presupposes variability (heterogeneity). The choice of a model was based on the Cochran homogeneity test (Q) (Cochran 1954), which follows a chi-square distribution with $(k-1)$ degrees of freedom:

$$Q = \sum_{j=1}^k w_j (\hat{\theta}_j - \hat{\theta}_m)^2$$

where $\hat{\theta}_j$ is the effect of the j th cutting ($j = 1, 2 \dots k$); $\hat{\theta}_m$ is the meta-analytical estimate (h^2 , μ , $\hat{\sigma}_p^2$ or $\hat{\sigma}_g^2$); and w_j is the weight of each harvest in the meta-analysis, which is given by the inverse of the variance of $\hat{\theta}_j$. The fixed effect model is given by $\hat{\theta}_j = \theta_m + \varepsilon_j$, where ε_j is the model error, $\varepsilon_j \sim N(0, w_j^{-1})$. Therefore, the maximum likelihood estimator of θ_m is given by $\hat{\theta}_{MV} = \frac{\sum_{j=1}^k w_j \hat{\theta}_j}{\sum_{j=1}^k w_j} \sim N\left(\theta_m, \frac{1}{\sum_{j=1}^k w_j}\right)$.

The random effects model is described as $\hat{\theta}_j = \theta_m + \delta_j + \varepsilon_j$, where δ_j is the set of random effects and ε_j is the residuals ($j = 1, 2 \dots k$); $\delta_j \sim N(0, \tau^2)$ and $\varepsilon_j \sim N(0, w_j^{-1})$ are considered to be independent of each other: $\hat{\theta}_j \sim N(\theta_m, \tau^2 + w_j^{-1})$, where τ^2 is the variability between cuttings that quantify the heterogeneity. The maximum likelihood estimator of θ_m is given by $\hat{\theta}_{MV} = \frac{\sum_{j=1}^k \hat{\theta}_j w_j^*}{\sum_{j=1}^k w_j^*}$, where $w_j^* = \frac{1}{\tau^2 + w_j^{-1}}$.

Meta-analysis was performed using the R script software (R Core Team 2020) integrated with the Genes software (Cruz 2016).

RESULTS AND DISCUSSION

Deviation analysis showed statistical significance ($p < 0.01$) for the genetic effect and permanent environmental effect by the Likelihood Ratio Test (-26.91 and -227.3, respectively), suggesting the presence of genetic diversity. Genetic diversity was also identified by Santos et al. (2020) for the same alfalfa germplasm. The importance of genetic diversity for plant breeding is based on the fact that crosses involving genetically different parents are better able to maintain

genetic variability in advanced generations (Cruz et al. 2011). This variability is especially important for alfalfa, which has severe sensitivity to depression due to inbreeding and exhibits self-incompatibility and autosterility (Kopp 2011, Santos et al. 2020). Individual estimates of μ , Pheno.V, Geno.V, Perm.Env.V, and h^2 for dry matter yield (DMY) are shown in Table 1. There was wide variation in the parameters along with the cuttings due to the environmental variations that are common in experiments of perennial crops (Hand and Crowder 2017), which leads to different gene expression.

The adjusted means for DMY ranged from 1.019,5 kg ha⁻¹ in the eighth harvest to 2.684,8 kg ha⁻¹ in the last one. The lowest yields occurred in cuttings 8, 20, 9, and 21, which did not receive irrigation. Accumulated DMY indicates that the germplasm has potential to integrate alfalfa base populations (Figure 1). Although DMY decreased in cuttings under water deficit, there was a rapid increase in DMY in accessions after irrigation was reestablished. Pheno.V ranged from 118.305,16 in harvest 5 to 518.035,4 in harvest 22 (Table 1). These estimates were affected by the environment, since large variations in temperature and rainfall were observed throughout the harvests (Figure 2). Geno.V also fluctuated along with the cuttings (24.560,1 to 149.568,2), due to differential gene expression. The variation in the accessions arose from high variance, due to the diversity of the accessions. Perm.Env.V ranged from 12.934,5 in the 13th harvest to 113.589,0 in the 24th. The permanent environmental effect is particular to longitudinal data and is estimated by the variance among harvests made on an individual in its plot. Genetic factors, such as dominance or epistatic effects, affect the permanent environmental effect, especially in early cuttings.

Broad sense heritability for DMY ranged from 9% to 57% (Table 1). In harvests where the heritability is high, there is better selection accuracy. Santos et al. (2018), investigating alfalfa performance under water deficit, stated that in harvests where a water deficit occurs, heritability increases. However, our results did not show the same pattern; h^2 estimates in harvests with water deficit ranged from 25% (harvest 10) to 35% (harvest 21). Additionally, there was a slight tendency of increase in h^2 over time. The highest h^2 for the first three harvests was 17%, and for the last three, 57%. This was mainly due to increased genetic variation. Genetic expression of the accessions increased over successive

Table 1. Estimates of genetic variance (Geno.V), permanent environmental variance (Perm.Env.V), phenotypic variance (Pheno.V), heritability (h^2), and mean of dry matter yield of 24 alfalfa harvest times regarding 77 accessions

Harvest	Geno.V	Perm.Env.V	Pheno.V	h^2 (%)	Mean
1	24560.1	27381.2	268746	9	2212.22
2	25201.8	23243.3	181615	14	2240.43
3	27597.5	20076.2	163884	17	1824.69
4	31338.1	17623.9	146692	21	1655.72
5	35945.4	15855	118305	30	1466.42
6	39360.4	14990.3	180549	22	1861.39
7	43025.2	14324.4	171956	25	1855.78
8	45234	13998	141962	32	1019.5
9	46851.3	13721.3	155786	30	1249.6
10	47402.6	13501.1	191084	25	1542.23
11	47264.5	13267.6	208101	23	1870.86
12	46186.3	12972.4	193064	24	2463.73
13	45370.3	12934.5	237150	19	2449.03
14	44758.3	13062.1	160837	28	1509.03
15	44261.2	13499.6	186264	24	2140.11
16	44183.1	14428.3	220754	20	2120.93
17	44732.7	15957.9	203495	22	1493.29
18	46805.8	19315.7	367642	13	2120.88
19	51288.4	25053.9	219488	23	2132.68
20	56169.3	30651.1	211438	27	1107.74
21	79074	53899.5	224230	35	1321.45
22	95083.4	68619	518035	18	2511.28
23	117122	87603.8	212982	55	2673.92
24	149568	113589	263157	57	2684.83

cuttings, indicating the necessity of evaluating an adequate number of harvests to observe the best expression of the genetic variability of the genotypes.

Having estimates for each harvest is extremely important because, from genetic parameters, it is possible to identify harvests where a major or minor variation occurs. The magnitude of genetic variances, for example, highlights the genetic

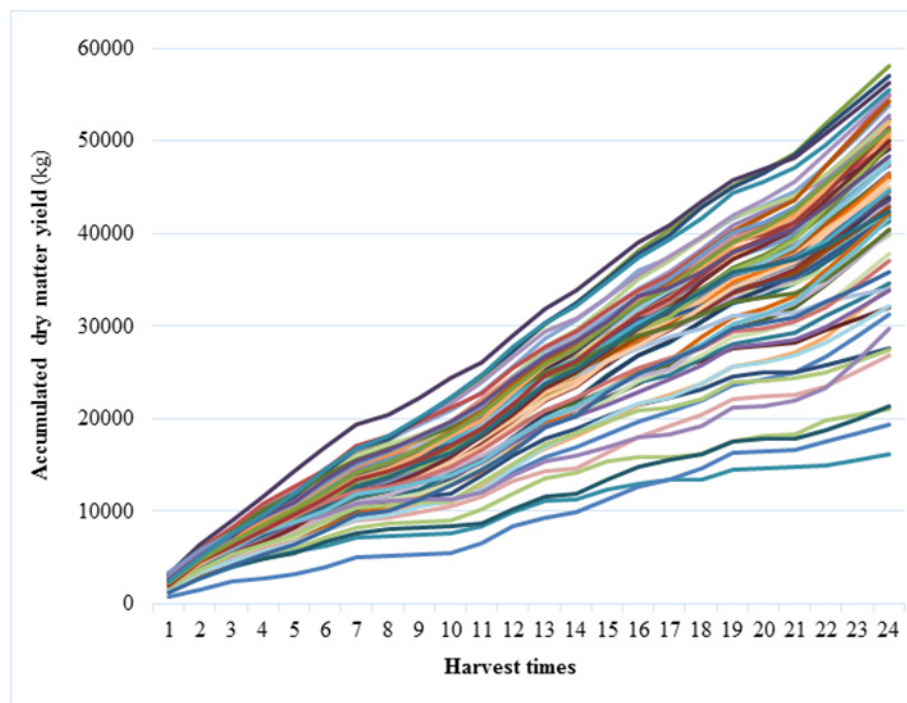


Figure 1. Accumulated dry matter yield of alfalfa over 24 harvest times.

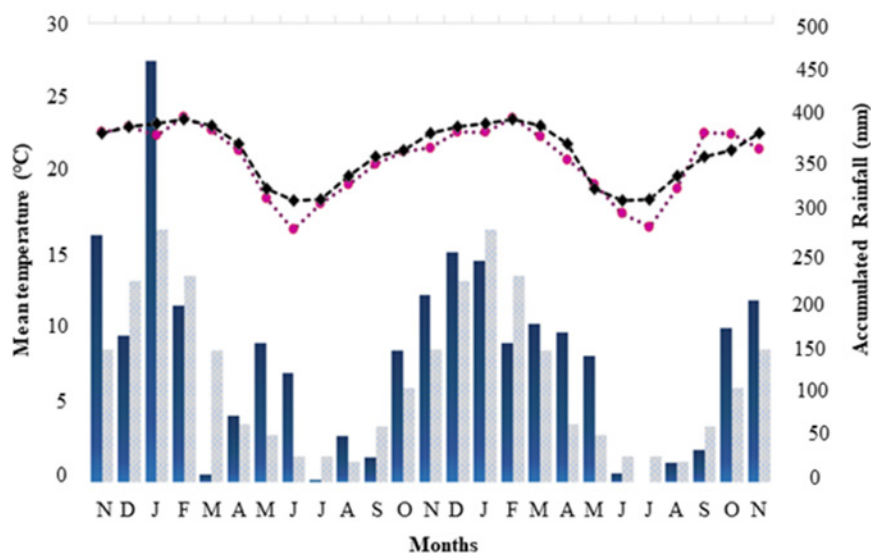


Figure 2. Meteorological data of São Carlos, São Paulo, Brazil. Mean temperature (dark blue bars) and accumulated rainfall (purple line) for the period from November 2015 to November 2017. Monthly averages obtained from the period of 1992 - 2010 shown on light blue bars (mean temperature) and black line (accumulated rainfall average).

differentiation among genotypes in each harvest. It is also possible to obtain trajectories over time. Since there is a lack of independence of residuals, the permanent environmental effect is included in the model. It should be noted that six alfalfa harvests ($r^2 = 0.95$) would be enough to estimate genetic parameters in this dataset.

However, a single meta-analytical estimate would provide an overview of the breeding potential of the germplasm. It would also be possible to identify the harvest in which selection would have the highest accuracy (Possobom et al. 2020). As seen in Table 1, and confirmed by the Cochran homogeneity test (Q) (Table 2), there is variability among the estimates of the parameters over the various harvests. Thus, meta-analytical estimates were obtained by the random model (Table 2). The estimates from the mixed model and meta-analysis approaches are not directly comparable because they are based on different ways of estimating parameters. An important difference between them is in regard to the presence of the permanent environmental effect in the former.

The mean DMY for the mixed model estimates was $\sim 1,866 \text{ kg ha}^{-1}$, similar to the average obtained by the meta-analysis ($1,819,71 \text{ kg ha}^{-1}$), considered excellent for this crop (Vasconcelos et al. 2008). In a study on alfalfa genotypes, Vasconcelos et al. (2008) found a mean yield of $1,475,29 \text{ kg ha}^{-1}$ in the nine most adapted alfalfa genotypes they evaluated. Nascimento et al. (2015) evaluated the adaptability of alfalfa genotypes through a Bayesian approach. They found that the genotype P 105 had the highest mean yield ($1,622,76 \text{ kg ha}^{-1}$). The mean estimate in our study was high compared to studies with other alfalfa genotypes.

Although both methods have similar mean DMY, the mixed model estimate was slightly higher, with a smaller standard deviation (43.19, compared to 98.45 for the meta-analytical estimate). Smaller standard deviations were also observed for Pheno.V, Geno.V, and h^2 in this approach. This result suggests the importance of including environmental effects in the model, because the particular conditions of each harvest have a major effect on estimation of parameters (Santos et al. 2018). While the mixed model averages of Pheno.V and Geno.V were 198,279,13 and 45,302,17, respectively, the meta-analytical estimates for Pheno.V and Geno.V were 108,345,82 and 63,680,65 (Table 2). Although there were differences in the estimates of the two approaches, both indicated the presence of genetic variance in comparable magnitudes. As phenotypic and genotypic variances are determinants of gain from selection, it is important to be aware of their values.

The average estimate of heritability for the mixed model was 24% (Table 2). This result is smaller than that reported by Santos et al. (2018) in a study on alfalfa accessions. The authors considered four harvests under specific edaphic and climatic conditions, leading to a higher correlation between the phenotypic value and the genotypic value (Cruz et al. 2012). The average of h^2 in the mixed model approach in this study revealed the obstacles of selecting alfalfa genotypes based on their phenotypic values. Higher estimates based on analysis of variance, for example, can allow breeders to avoid wrong decisions. The meta-analytical estimate for heritability (57.3%) suggested that the alfalfa accessions had satisfactory genetic variability in relation to the total variability. However, this estimate has to be carefully interpreted, since the environmental effect is not present in this approach. According to Borem et al. (2017), parents with high-performance tend to produce high-performance progenies if these accessions are used in crossing blocks to form synthetic alfalfa populations. The establishment of base populations should consider selection of individuals based on DMY, which provides substantial gain in the initial phase of alfalfa breeding programs.

Weighted meta-analysis and mixed models in forage breeding programs

Evaluation of the genetic potential of germplasm in perennial crops is affected by the characteristics of longitudinal data, such as the correlation between harvests. Combined estimates should be carefully evaluated for each purpose.

Table 2. Average estimates obtained from the 24 harvest times considering the mixed model approach (\bar{X}), meta-analytical estimates ($\hat{\theta}_m$), and Cochran homogeneity test (Q) for the average (μ), phenotypic variance (Pheno.V), genotypic variance (Geno.V), and heritability (h^2)

Parameters	\bar{X}	$\hat{\theta}_m$	Homogeneity Test (Q)
μ	1866.13 (± 43.19)	1819.71 (± 98.45)	11744.1***
Pheno.V	198279.13 (± 445.28)	108345.82 (± 10082.30)	226.129***
Geno.V	45302.17 (± 212.84)	63680.65 (± 8758.22)	153.291***
h^2	24 (± 48.54)	57.32 (± 3.80)	387.362***

***Significant at the 0.001 probability level.

Meta-analytical estimates, on the other hand, represent the common conditions for alfalfa experiments due to the different weights given according to the variation in each estimate. This variation, associated with an accurate measurement, reflects the experimental conditions, the experimental design, the variation in harvests or environments, etc.

Estimating individual genetic parameters is an effective and more detailed approach for capturing trends. However, meta-analysis (when properly performed) has also proven to be effective, since the mean and genetic variance had values of the same magnitude (mean of 1.866,13 in the mixed model compared to 1.819,71 in the meta-analysis, and genetic variance of 45.302,17 in the mixed model compared to 63.680,65 in the meta-analysis). The efficiency of meta-analysis is shown in variations in the estimates of Geno.V and h^2 . The standard deviation for the random effects estimates in the meta-analysis was greater than the standard deviation for the mixed model. This can be explained by the estimation method used in the random model, which considers the existence of heterogeneity among harvests. Despite their differences, the results obtained by the mixed model analysis and meta-analysis can be complementary.

Information coming from mixed-model and meta-analysis can be analyzed from different perspectives to provide distinct insights to the breeder. The mixed model approach allows breeders to check for the existence of variability among genotypes, as well as for the genotype-by-environment interaction. The effects of this interaction are one of the main challenges faced by breeders at the time of identification, selection, and recommendation of cultivars (Gonçalves et al. 2020). Genotypic variance and the genotype-by-environment interaction are particularly important in recognizing the best genotypes. They have been used to set up breeding strategies, through the existence of variability, and to rank genotypes, based on predicted values. Through a meta-analysis approach, breeders can observe the results of a given experiment after assembling a wide range of information over time. The final information can guide or adjust new management practices and experimental strategies, and allow judgment of a particular experiment based on knowledge generated by a meta-analysis that uses the information from experiments conducted in a similar way.

It is reasonable to affirm that the potential of alfalfa germplasm at any cutting can be determined based on the combined estimates obtained by meta-analysis. However, extra care is necessary when selecting accessions under discrepant conditions, e.g., in the presence of interaction. In this case, parameter estimates may not reflect reality (Fernandes et al. 2017).

The germplasm of this study showed potential for use in composing base populations in alfalfa breeding programs. The germplasm has high mean DMY and high genetic variability, which make the choice of parents for future crosses easier and suggest that the selection strategy will be successful. Santos et al. (2020) clustered the accessions of this study based on phenotypic and molecular data and suggested a promising base population would be composed of Pro INTA Patricia, Pro INTA Super Monarca, Mecha, Magna 601, WL 525, ACA 900, Bacana, CUF 101, Crioula, and Ruano. The authors stated the importance of having a broad base population with favorable alleles for regrowth ability, biomass yield, and persistence.

It should be noted that forage breeding has objectives similar to those of breeding cereal crops: higher yield, resistance to pests and diseases, production of viable seeds, efficient use of fertilizers, and adaptation to biotic stresses. However, for forages, persistence and nutritional value play a key role. As a result, plant breeders opt for a breeding program based on yield and nutritional quality, usually expressed by plant chemical composition and its correlations with digestibility and consumption. Future studies should be based on accessions that have these qualities. Due to the presence of a complex genomic structure, severe inbreeding depression, and cross-pollination (Nagl et al. 2011), the correct choice of parents for alfalfa poly-crossing is fundamental for genetic progress of the crop (Santos et al. 2020). To compose crosses that will form synthetic populations of high dry matter yield, for example, it is necessary to choose high yielding accessions, such as those from the germplasm used in this study.

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